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1 NETWORKED2A, an actin-membrane adaptor,  
2 binds specific protein kinases at the pollen tube  
3 plasma membrane.

4

5 **Actin-Membrane Interactions Mediated by**  
6 **NETWORKED2 in Arabidopsis Pollen Tubes**  
7 **Through Associations with Pollen Receptor-**  
8 **Like Kinase 4 & 5.**

9

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29 Figures: 6 figures (all to be reproduced in colour).

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## Summary

34

35       • During fertilisation, Pollen Receptor-Like Kinases  
36       (PRKs) control pollen tube growth through the  
37       pistil in response to extracellular signals, and  
38       regulate the actin cytoskeleton at the tube apex to  
39       drive tip growth.

40

41       • We investigated a novel link between membrane-  
42       integral PRKs and the actin cytoskeleton,  
43       mediated through interactions between PRKs and  
44       NET2A; a pollen-specific member of the  
45       NETWORKED superfamily of actin-binding  
46       proteins.

47

48       • We characterise NET2A as a novel actin-  
49       associated protein that localises to punctae at the  
50       plasma membrane of the pollen tube shank, which  
51       are stably associated with cortical longitudinal  
52       actin cables. NET2A was demonstrated to interact  
53       specifically with PRK4 and PRK5 in *Nicotiana*  
54       *benthamiana* transient expression assays, and  
55       associated at discrete foci at the shank membrane  
56       of Arabidopsis pollen tubes. Our data indicates  
57       NET2A is recruited to the plasma membrane by  
58       PRK4 and PRK5, and that PRK kinase activity is  
59       important in facilitating its interaction with NET2A.

60

61       • We conclude that NET2A-PRK interactions  
62       mediate discrete sites of stable interactions  
63       between the cortical longitudinal actin cables and  
64       plasma membrane in the shank region of growing

65 pollen tubes, which we have termed Actin-  
66 Membrane Contact Sites (AMCSs). Interactions  
67 between PRKs and NET2A implicate a role for  
68 NET2A in signal transduction to the actin  
69 cytoskeleton during fertilisation.

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## 72 **Keywords**

73 Actin, Cytoskeleton, Fertilisation, Membrane,  
74 NET2A, Pollen, PRK, Signalling.

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Pollen tube growth is a critical step of fertilisation during the angiosperm reproductive cycle, and facilitates the delivery of non-motile sperm cells to the female gamete. It is known that the growing tube is guided through the pistil to the ovules by a large number of secreted signalling molecules, to ensure the targeting of pollen tube growth to the egg (Qu *et al.*, 2015a); however our knowledge of the mechanisms controlling pollen tube growth and guidance during fertilisation remain limited.

The actin cytoskeleton is crucial for pollen tube growth (Gibbon *et al.*, 1999; Vidali *et al.*, 2001); driving cytoplasmic streaming (Vidali *et al.*, 2001) and targeting of Golgi-derived secretory vesicles to the growing tip (Vidali & Helper, 2001; Lee *et al.*, 2008; Rounds *et al.*, 2014), whilst actin-dependent exocytosis and endocytosis also occurs in the pollen tube shank region (Moscatelli *et al.*, 2012). To achieve polarised cell growth, the actin cytoskeleton has a highly organised and distinctive structure in growing pollen tubes. In the shank region of the tube, (corresponding to the non-growing region, >4  $\mu\text{m}$  from the tip; Qu *et al.*, 2017), filamentous actin (F-actin) is arranged into thick longitudinal actin cables, coordinating rapid, long range transport of organelles (Chen *et al.*, 2009; Qu *et al.*, 2015b). At the apical zone, (corresponding to the growing region, <4  $\mu\text{m}$  from the tip; Qu *et al.*, 2017), a distinct and highly dynamic population of longitudinally-aligned actin filaments coordinate tip growth and turning: cortical filaments drive and define the direction of tip growth through targeted apical exocytosis, and cytoplasmic filaments prevent retrograde movement of vesicles (Kost *et al.*, 1999, Lovy-Wheeler *et al.*, 2005; Lee *et al.*, 2008; Chen *et al.*, 2009; Qu *et al.*, 2017). This

122 highly distinctive actin structure is regulated by a large  
123 number of actin-binding proteins, which regulate actin  
124 dynamics and organisation (Hussey *et al.*, 2006; Staiger  
125 *et al.*, 2010; and Qu *et al.*, 2015b).

126 During fertilisation, the pollen tube actin cytoskeleton  
127 must be regulated in response to extracellular signals to  
128 drive pollen tube growth and navigation in the pistil. The  
129 actin cytoskeleton of pollen tubes is regulated by Pollen  
130 Receptor-Like Kinases (PRKs); a family of  
131 transmembrane leucine-rich repeat (LRR) receptor-like  
132 kinases (RLKs), with important roles in fertilisation (Lee *et al.*, 1996; Takeuchi & Higashiyama, 2016). PRKs are  
133 known to influence pollen tube growth (Chang *et al.*,  
134 2013), downstream of binding external signalling ligands  
135 (Tang *et al.*, 2002; Tang *et al.*, 2004; Wengier *et al.*,  
136 2010; Huang *et al.*, 2014) and mediate pollen tube  
137 navigation towards pistil-secreted guidance cues  
138 (Takeuchi & Higashiyama, 2016), demonstrating their  
139 importance as upstream surface regulators of pollen tube  
140 growth. PRKs have been implicated as regulators of the  
141 actin cytoskeleton through their involvement with Rop  
142 (Rho of plants) GTPases; molecular switches that control  
143 tip extension through the ROP-interactive CRIB-  
144 containing protein 3 (RIC3)/RIC4 pathway, which co-  
145 ordinates actin dynamics at the pollen tube apex (Fu *et al.*, 2001; Gu *et al.*, 2005; Zhang & McCormick, 2007; Lee  
146 *et al.*, 2008; Chang *et al.*, 2013, Takeuchi & Higashiyama,  
147 2016). Therefore, PRKs are thought to control pollen tube  
148 growth downstream of external guidance signals through  
149 regulation of actin at the tube apex. However, the  
150 mechanisms of signal transduction to the pollen tube  
151 actin cytoskeleton by PRKs are only recently becoming  
152 understood, and it is likely that novel regulatory links

155 between PRKs and actin have yet to be discovered.  
156 Moreover, these cited studies have focused on the  
157 coupling of actin dynamics to the growing plasma  
158 membrane and trafficking at the tip, but have not revealed  
159 how villin and fimbrin-bundled actin of the shank  
160 interfaces with the older membrane and maturing cell  
161 wall.

162 Here, we report the identification of a novel link between  
163 PRK membrane receptors and the actin cytoskeleton,  
164 mediated by the actin-binding NET2 proteins. The NET2  
165 proteins are a pollen-expressed subclade of the  
166 NETWORKED superfamily of actin-binding proteins,  
167 which bind actin filaments at various membrane  
168 compartments through their conserved N-terminal NAB  
169 (NET actin-binding) domains (Deeks *et al.*, 2012; Wang  
170 *et al.*, 2014). Members of the NET2 subfamily localise to  
171 discreet foci at the plasma membrane of the pollen tube  
172 shank, at which they bind both integral membrane protein  
173 kinases, PRK4 and PRK5, and cortical longitudinal actin  
174 cables. Furthermore, these results indicate that the NET2  
175 proteins are regulated by PRKs to mediate stable points  
176 of contact between the plasma membrane and actin  
177 filaments in the pollen tube shank, which we have termed  
178 'actin-membrane contact sites (AMCS)'.

179 Our data identify a role for NET2A in forming links with  
180 specific PRKs, raising the possibility that this connection  
181 at the AMCS acts as a platform for the transduction of  
182 extracellular signals to the actin cytoskeleton during  
183 fertilisation.

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## Materials and Methods

### Plant material and transformations

*Arabidopsis thaliana* (L.) Heynh. (col-0) ecotype was used for the generation of stable *Arabidopsis* transformants using the floral dipping method according to Zhang *et al.*, (2006). Seeds were grown on ½ Murashige & Skoog (MS) agar or compost in a growth chamber with a 16-hour day and 8-hour night cycle, with 22 °C day temperature and 18 °C night temperature.

Transient transformation of *Nicotiana benthamiana* was performed using leaf infiltration as described Sparkes *et al.*, (2006). Plants were grown in a growth chamber with a 16-hour day and 8-hour night cycle, with 25 °C day temperature and 18 °C night temperature.

### Molecular cloning and vectors

cDNAs of full-length NET2A, NET2B, PRK1, PRK2, PRK3, PRK4, PRK5, PRK6, were amplified from total floral cDNA using polymerase chain reaction (PCR), with the primers listed in table S1. Coding sequences of respective subdomains and truncations of these proteins were also amplified from these cDNA templates using the primers list in table S1. The cDNAs were transiently expressed in *N. benthamiana* leaf epidermal cells as fluorescent fusion proteins by cloning them into various binary gateway vectors using the gateway cloning system (Invitrogen). pB7FGW2 for C-terminal green fluorescent protein (GFP), pH7RGW2 for C-terminal red fluorescent protein (RFP) and pMDC83-mCherry for (C-terminal mCherry) were used.



218 For stable expression of PRK4 and PRK5 as fluorophore  
219 fusions under the *pLAT52* promoter, pB7FGW52 (C-  
220 terminal GFP) and pH7RGW52 (C-terminal RFP) were  
221 used.

222 The expression vectors pMDC83-mCherry, pB7FGW52  
223 and pH7RGW52 were generated using restriction  
224 subcloning. To generate pMDC83-mCherry, the mCherry  
225 coding sequence was PCR amplified with added 5'*AscI*/  
226 and 3'*BstBI* restriction sites using the primers listed in  
227 table S1. *AscI*/*BstBI* double restriction digest of pMDC83  
228 was performed to excise the GFP coding sequence, and  
229 ligation of 5'-*AscI*-mCherry-*BstBI*-3' into the pMDC83  
230 *AscI*/*BstBI* site was performed using T7 DNA ligase  
231 (NEB). To generate pB7FGW52 and pH7RGW52, the  
232 *pLAT52* promoter sequence (Twell *et al.*, 1990) was PCR  
233 amplified with added 5'*SacI* and 3'*SpeI* sites using the  
234 primers described in table S1. Excision of the *CaMV* 35s:  
235 promoter sequence was performed using *SacI*/*SpeI*  
236 double restriction digest, and the 5'-*SacI*-*pLAT52*-*SpeI*-3'  
237 DNA fragment was ligated into the excision site using T7  
238 DNA ligase (NEB).

239 To generate the PRK5<sup>K403R</sup> kinase-dead PRK5 mutant  
240 construct (in which Lysine-403 of PRK5 was mutagenised  
241 to Arginine), site-directed mutagenesis was performed on  
242 the full-length, wild-type PRK5 coding sequence using the  
243 QuickChange II Site Directed Mutagenesis Kit (Agilent).  
244 The codon for Lysine-403 was altered to Arginine using  
245 the primers listed in table S1.

246

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249     **Live cell imaging and FRET-FLIM**

250     Transiently transformed *N. benthamiana* leaves were  
251     imaged 4 days after infiltration using laser scanning  
252     confocal microscopy (LSCM; Leica TCS SP5). Images  
253     were acquired in multi-track mode with line switching  
254     when imaging co-localisation of multiple fluorophores. For  
255     drug treatments, leaf sections were incubated in 50  $\mu$ M  
256     Latrunculin B (30 minutes) or 50  $\mu$ M amiprophos methyl  
257     (APM; 2 hours) to disrupt actin or microtubules  
258     respectively.

259     Förster resonance energy transfer-fluorescence lifetime  
260     imaging microscopy (FRET-FLIM) was performed using  
261     the Leica TCS SP5 SMD LSCM combined with  
262     fluorescence lifetime system (PicoQuant). Data analysis  
263     and acquisition was performed with SymPhoTime  
264     software (PicoQuant). The lifetime of the donor construct  
265     expressed alone was measured as a negative control,  
266     and compared to the lifetime of the donor when co-  
267     expressed with the acceptor construct. The GFP  
268     fluorescence lifetimes of GFP-RFP and GFP-mCherry  
269     fusion proteins were measured as a positive control. All  
270     measurements were taken from whole-field images of  
271     cells expressing fluorophore fusion proteins at similar  
272     levels.

273

274     **Yeast-2-hybrid (Y2H)**

275     The intracellular domains of PRK1, PRK2, PRK3, PRK4,  
276     PRK5 and PRK6 were PCR amplified using the primers  
277     listed in table S1. The cDNAs were cloned into pGBKT7  
278     (Clontech) using gateway cloning (Invitrogen), to facilitate  
279     their expression as bait protein constructs. The full-length  
280     NET2A cDNA was cloned into pGADT7 (Clontech) using

281 the gateway cloning system (Invitrogen) to facilitate its  
282 expression as prey protein constructs.

283 The pGBKT7 constructs were transformed into the MAT $\alpha$   
284 *Saccharomyces cerevisiae* strain Y187 (Clontech), and  
285 pGADT7 constructs were transformed into the MAT $\alpha$   
286 strain, AH109 (Clontech) using the manufacturer's  
287 instructions.

288 NET2A in pGADT7 was mated against each pGBKT7  
289 construct on yeast peptone dextrose adenine (YPDA)  
290 media at 28 °C for 24 hours, and diploids containing both  
291 constructs were selected on standard defined (SD) media  
292 lacking Leucine and Tryptophan. Interactions between  
293 bait and prey protein constructs was assessed by  
294 selecting diploid yeast on SD media also lacking  
295 Histidine, and supplemented with 2.5 mM 3-Amino-1,2,4-  
296 triazole (3AT). As negative controls, pGADT7 constructs  
297 were mated against empty pGBKT7, and pGBKT7  
298 constructs were mated against empty pGADT7.

299

### 300 ***In vitro* pollen germination and observation**

301 Arabidopsis pollen was germinated *in vitro* on solid  
302 germination media as described by Li *et al.*, (1999).  
303 Germination media consisted of 18 (w/v) % sucrose, 0.01  
304 % (w/v) H<sub>3</sub>BO<sub>4</sub>, 1 mM Ca(NO<sub>3</sub>)<sub>2</sub>, 1 mM MgSO<sub>4</sub>, 1 mM  
305 CaCl<sub>2</sub>, and 0.5 % (w/v) Agarose Type VII-A (Sigma), pH  
306 7. Mature Arabidopsis pollen was dusted onto the solid  
307 germination media. 3 - 4 excised Arabidopsis pistils were  
308 placed on surface of the media and samples were  
309 incubated in a dark humid environment at 22 °C for > 4  
310 hours. Subsequently, germinated pollen was analysed  
311 using LSCM as described above.

## Results

### **All members of the Arabidopsis NET2 subfamily co-localise with actin filaments *in vivo***

The NET proteins represent a novel superfamily of actin-binding proteins which we have shown to associate with actin through their conserved N-terminal NAB domains (Deeks *et al.*, 2012; Wang *et al.*, 2014). Accordingly, the NAB domain is highly conserved in each member of the NET2 subclade (Fig. 1a; Hawkins *et al.*, 2014), indicating that they are also likely to bind actin directly. Here, we show each member of the NET2 subfamily has the ability to associate with F-actin *in vivo*. GFP fusions of the NET2A NAB domain were observed to localise to actin filament networks when transiently expressed in *N. benthamiana* (a simple experimental system for rapid expression and analysis of fluorescently-tagged proteins). NET2A-GFP co-localised with the F-actin marker, RFP-lifeact (Fig. 1b), and this localisation was disrupted by treatment with actin-targeting drugs (Fig. 1c). Likewise, GFP fusions of the NET2B, NET2C and NET2D NAB domains also localised to actin filaments *in vivo* (Fig. 1d), effectively demonstrating each NET2 subfamily member can localise to F-actin through their N-terminal NAB domains. It was observed that full-length NET2A-GFP and NET2B-GFP also localised to actin filaments when transiently expressed in *N. benthamiana* leaves:  $90.7 \pm 2.3$  % NET2A-GFP punctae co-localised with actin filaments, decorating them in the 'beads-on-a-string pattern', as is characteristic of NET superfamily proteins (Fig. 1e, Fig. S1; Deeks *et al.*, 2012). Taken together, our data indicates that each member of the NET2 subclade is

344 able to localise to F-actin *in vivo*, through their N-terminal  
345 NAB domains.

346

347 **NET2A co-localises with F-actin at the pollen tube**  
348 **plasma membrane**

349 Having determined the ability of the NET2 proteins to  
350 localise to the actin cytoskeleton in transient leaf  
351 transformation, it was then investigated as to whether  
352 they may also co-localise with actin filaments *in situ*.  
353 Therefore, we analysed NET2A-GFP in Arabidopsis  
354 pollen tubes (the NET2 proteins' endogenous  
355 environment). Previously, we have demonstrated that  
356 native promoter-driven NET2A-GFP localises to discrete  
357 punctae specifically at the shank region of the pollen tube  
358 plasma membrane (Deeks *et al.*, 2012; Fig. 2a & 2b).  
359 Here, we demonstrate that these NET2A foci co-localise  
360 with cortical F-actin cables at the shank membrane of the  
361 pollen tube. The NET2A-GFP punctae aligned along actin  
362 cables stained with the F-actin probe, rhodamine-  
363 phalloidin (Fig. 2c), and co-localised with F-actin  
364 filaments in live pollen tubes co-expressing native  
365 promoter-driven NET2A-GFP and the genetically  
366 encoded actin-marker construct, FABD2-RFP, stably  
367 expressed in pollen under the pollen-specific promoter,  
368 *pLAT52* (Fig. 2d; Twell *et al.*, 1990). The NET2A punctae  
369 decorated actin filaments in the characteristic 'beads-on-  
370 a-string' pattern typical of NET superfamily proteins, and  
371  $80.2 \pm 6.1$  % of NET2A-GFP punctae were observed to  
372 co-localise with FABD2-RFP-labelled actin filaments.  
373 Using rapid time-lapse imaging, we observed the  
374 localisation of NET2A-GFP punctae at the plasma  
375 membrane to be highly stable and persist at the

376 membrane throughout pollen tube growth (video S1). The  
377 punctae were not highly motile, but appeared to undergo  
378 abrupt, co-ordinated, short-range, anterograde and  
379 retrograde movements along linear vectors (Fig. S2). This  
380 indicates that NET2A localises to stable punctae at the  
381 pollen tube cortex. Taken together, these data show that  
382 NET2A forms stable associations with cortical actin  
383 filaments at the pollen tube membrane.

384

### 385 **NET2A interacts specifically with PRK isoforms 4 and** 386 **5**

387 Our data showed that F-actin localisation is conferred by  
388 the NAB domains of NET2 proteins, however it remained  
389 unknown how actin-localised foci of full-length NET2A are  
390 recruited to the plasma membrane as NET proteins do  
391 not contain transmembrane domains or identifiable  
392 modification sites associated with known peripheral  
393 membrane proteins. A potential orthologue of the NET2  
394 proteins in *Petunia*, *Petunia inflata* Kinase Interacting  
395 Protein 1 (PiKIP1), has been identified as an interactor of  
396 PRK proteins in a Y2H screen using *Petunia inflata*  
397 Pollen Receptor-Like Kinase 1 (PiPRK1) as bait (Skirpan  
398 *et al.*, 2001). Importantly, PiKIP1 was not characterised  
399 as a NET-family actin-binding protein. PRKs are integral  
400 membrane proteins, suggesting the hypothesis that PRKs  
401 contribute to NET2 membrane recruitment. We used  
402 combinatorial Y2H to test the potential for interactions  
403 between Arabidopsis NET2 and PRK family members.  
404 Full-length NET2A was observed to interact with the  
405 cytosolic domains of PRK4 and PRK5 (Fig. 3a) but did  
406 not interact with PRK1, PRK2, PRK3 or PRK6 (Fig. S3).  
407 Interestingly, PRK4 and PRK5 belong to a distinct

408 evolutionary subclade of PRKs (Chang *et al.*, 2013;  
409 Takeuchi & Higashiyama., 2016), suggesting that the  
410 NET2 family show sequence-based isoform specificity in  
411 this assay.

412 We then sought to validate NET2 kinase interactions *in*  
413 *planta* using FRET-FLIM, NET2A-mCherry interacted  
414 specifically with PRK4-GFP and PRK5-GFP in FRET-  
415 FLIM assays when transiently expressed in *N.*  
416 *benthamiana* leaf tissue. When co-expressed with  
417 NET2A-mCherry, the average fluorescence lifetime of  
418 PRK4-GFP was reduced by 0.23 ns to  $2.22 \pm 0.06$  ns  
419 compared to the control ( $2.45 \pm 0.02$  ns). Similarly, the  
420 fluorescence lifetime of PRK5-GFP was reduced by 0.36  
421 ns to  $2.15 \pm 0.02$  ns compared to the control ( $2.51 \pm 0.02$   
422 ns; Fig. 3b), sufficient to demonstrate an interaction  
423 (Danquah *et al.*, 2011; Wang *et al.*, 2014). Consistent  
424 with the Y2H data, NET2A-mCherry did not interact with  
425 PRK1-GFP, PRK2-GFP, PRK3-GFP or PRK6-GFP (table  
426 S2). Interestingly, we also observed NET2B to interact  
427 specifically with PRK4 and PRK5 using FRET-FLIM (table  
428 S3). Our data therefore shows that multiple NET2  
429 subfamily members interact specifically with the  
430 PRK4/PRK5 subclade of Arabidopsis PRKs *in planta*.

431

432 **NET2s are recruited to the plasma membrane by**  
433 **PRK4 and PRK5.**

434 Transient co-expression of NET2A-GFP with either  
435 PRK4-RFP or PRK5-RFP in *N. benthamiana* leaves  
436 resulted in striking changes in NET2A-GFP subcellular  
437 localisation. Whereas NET2A-GFP localised to punctae  
438 and filaments when expressed alone, it was found  
439 distributed exclusively at the plasma membrane when co-

440 expressed with PRK5-GFP (Fig. 4a, Fig. S4); where the  
441 two proteins could be observed to co-localise (Fig. 4b).  
442 When co-expressed with PRK4-RFP, NET2A-GFP  
443 localised to the plasma membrane and peripheral cytosol  
444 (Fig. 4a). As a negative control, the subcellular  
445 localisation of NET2A-GFP was analysed when co-  
446 expressed with PRK6-RFP (no interactions between  
447 NET2A and PRK6 were detected in Y2H or FRET-FLIM  
448 assays; Fig. S3, Table S2). Importantly, NET2A-GFP was  
449 observed to remain localised to filaments and punctae  
450 and did not localise to the plasma membrane (Fig. S5).  
451 Furthermore, it was also observed that like NET2A-GFP,  
452 NET2B-GFP could also be recruited to the plasma  
453 membrane by PRK4-RFP and PRK5-RFP specifically  
454 (Fig. S6).

455 To further investigate how PRK4 and PRK5 interact with  
456 NET2 proteins, we analysed the specific subdomains of  
457 the PRKs that mediate the interaction with NET2A.  
458 Truncated PRK mutants lacking intracellular C-terminal  
459 kinase domains (PRK $\Delta$ K) were generated (Fig. 4c). RFP  
460 fusions of PRK4 $\Delta$ K (PRK4<sup>1-374</sup>) and PRK5 $\Delta$ K (PRK5<sup>1-376</sup>)  
461 were unable to recruit NET2A-GFP to the plasma  
462 membrane, which instead localised to punctae and  
463 filaments in a similar manner to NET2A-GFP expressed  
464 alone (Fig. 4d). FRET-FLIM indicated no interaction  
465 between NET2A-GFP and PRK5 $\Delta$ K-RFP (Fig. 4e),  
466 suggesting that PRKs bind and recruit NET2 proteins to  
467 the membrane through their cytoplasmic kinase domain.

468 We then investigated specific residues of PRK5 important  
469 in mediating the interaction with NET2A. *in vitro*  
470 experiments have indicated that phosphorylation of  
471 petunia PiKIP1 by PiPRK1 contributes to the interaction  
472 between the two proteins, and kinase-dead mutant



473 variants of PiPRK1 are diminished in their ability to bind  
474 PiKIP1. Lysine-403 of PRK5, (homologous to PiPRK1  
475 Lysine-462; predicted to be important for kinase  
476  $Mg^{2+}$ /ATP binding; Skirpan *et al.*, 2001) was replaced by  
477 Arginine to generate PRK5<sup>K403R</sup>. It was observed that the  
478 PRK5<sup>K403R</sup>-RFP construct recruited NET2A-GFP to the  
479 plasma membrane when co-expressed in *N.*  
480 *benthamiana* leaf epidermal cells, similar to WT PRK5-  
481 RFP. However, PRK5<sup>K403R</sup>-RFP showed reduced  
482 resonance with NET2A-GFP in the FRET-FLIM system  
483 (Fig. 4g). When co-expressed, the full length PRK5-RFP  
484 construct induced a decrease in average NET2A-GFP  
485 fluorescence lifetime of 0.38 ns to  $2.10 \pm 0.07$  ns,  
486 compared to the control ( $2.48 \pm 0.08$  ns). In comparison,  
487 PRK5<sup>K403R</sup>-RFP induced only a small decrease in  
488 average NET2A-GFP fluorescence lifetime of 0.14 ns to  
489  $2.34 \pm 0.05$  ns, suggestive of a relatively weak  
490 interaction. This indicates that Lysine-403 of PRK5 is  
491 important in facilitating the interaction between  
492 PRK4/PRK5 and NET2s *in vivo*. We speculate that  
493 PRK5 Lysine-403 is functionally equivalent to PiPRK1  
494 Lysine-462 and may be important for PRK5 kinase  
495 activity, which is likely to mediate an interaction with  
496 NET2A.

497 Taken together, the data suggests that specific members  
498 of the PRK family, namely PRK4 and PRK5, are able to  
499 bind, and recruit NET2 proteins to the plasma membrane  
500 *in vivo* through their intracellular kinase domains.

501

502

503

504 **NET2A associates with PRK4 and PRK5 at discreet**  
505 **foci at the plasma membrane of the pollen tube shank**

506 We have shown that NET2 proteins associate with actin  
507 filaments and can be recruited to the plasma membrane  
508 through interactions with specific PRKs in leaf transient  
509 expression assays. However, NET2A forms punctae at  
510 the plasma membrane of the pollen tube shank. We  
511 therefore asked whether populations of PRK4 and PRK5  
512 coincide with these punctae in growing pollen tubes. We  
513 observed PRK4-GFP and PRK5-GFP localised to  
514 discreet foci at the pollen tube plasma membrane (Fig. 5),  
515 with a similar pattern: the average puncta size for both  
516 PRK4-GFP and PRK5-GFP was observed to be highly  
517 similar (average PRK4-GFP puncta size =  $0.47 \pm 0.11$   
518  $\mu\text{m}$ , average PRK5-GFP puncta size =  $0.46 \pm 0.10$   $\mu\text{m}$ ),  
519 as was the density of PRK4-GFP and PRK5-GFP  
520 punctae at the shank plasma membrane (PRK4-GFP  
521 punctae density =  $0.65/\mu\text{m}^2$ , PRK5-GFP punctae density  
522 =  $0.62/\mu\text{m}^2$ ). The PRK4-GFP and PRK5-GFP punctae  
523 were, alike, distributed along the membrane of the pollen  
524 tube shank region but were reduced in intensity at the  
525 growing tip (both were visible only at distances greater  
526 than  $\approx 15$   $\mu\text{m}$  distal to the apex), in a manner highly  
527 similar to those of NET2A-GFP (Fig. 2). Therefore, it was  
528 investigated as to whether NET2A may associate with  
529 PRK4 and PRK5 at these membrane foci. The results  
530 show that NET2A-GFP and PRK4-RFP co-localise to the  
531 same punctae at discreet foci at the pollen tube  
532 membrane in stable transgenic Arabidopsis lines  
533 expressing native promoter-driven NET2A-GFP and  
534 PRK4-RFP (Fig. 6). In pollen tubes co-expressing  
535 NET2A-GFP and PRK4-RFP under *pLAT52*, we  
536 observed  $83.0 \pm 7.3$  % of NET2A-GFP punctae co-

537 localised with PRK4-RFP punctae (n = 265 punctae in 6  
538 cells). Taken together with the yeast 2-hybrid and FRET-  
539 FLIM experiments, these data show that NET2A co-  
540 localises with PRK4/PRK5 punctae at the pollen tube  
541 membrane, representing discrete sites of interaction  
542 between NET2A and PRK proteins at the plasma  
543 membrane of the pollen tube shank.

544

545

## 546 **Discussion**

547 Our data demonstrates a novel mechanism of interaction  
548 between the actin cytoskeleton and the pollen tube  
549 plasma membrane, in which NET2 proteins bind actin  
550 filaments to the plasma membrane through association  
551 with the membrane-integral pollen receptor-like kinases,  
552 PRK4 and PRK5. This discovery suggests that the NET2  
553 proteins have an important role in angiosperm fertilisation  
554 and in the regulation of the actin cytoskeleton in response  
555 to extracellular signals. In this context, whilst it is known  
556 that PRKs control actin dynamics at the pollen tube apex  
557 (Zhang & McCormick, 2007; Lee *et al.*, 2008; Chang *et*  
558 *al.*, 2013; Takeuchi & Higashiyama, 2016), nothing is  
559 known about how the cortical longitudinal actin cables of  
560 the pollen tube shank may be regulated at the plasma  
561 membrane in response to external signals. This unique  
562 subpopulation of actin filaments has specialised functions  
563 in mediating rapid, long-range anterograde, cytoplasmic  
564 streaming (Chen *et al.*, 2009; Qu *et al.*, 2015b), and their  
565 specific association with NET2A indicates importance of  
566 their regulation in response to external signals, and an  
567 interesting role for NET2 proteins in their organisation  
568 downstream of PRK signalling.

569 The NET2 proteins represent a subclade of the  
570 NETWORKED superfamily of actin-binding proteins,  
571 which associate with actin filaments at various organelle  
572 membranes through their N-terminal NAB domains  
573 (Deeks *et al.*, 2012). Accordingly, we have demonstrated  
574 that the NET2 proteins are, likewise, proteins that co-  
575 localise with F-actin *in vivo* through their conserved NAB  
576 domains, as GFP fusions of each NET2 NAB domain and  
577 full-length NET2 proteins were observed to localise to  
578 actin filaments *in vivo*. Consistent with other NET  
579 superfamily proteins, we show members of the NET2  
580 subfamily to bind actin at cellular membranes: NET2A  
581 was observed to localise to discrete foci at the pollen tube  
582 plasma membrane, which aligned along actin-filaments.  
583 Taken together, we conclude that NET2A associates with  
584 cortical actin at the plasma membrane of the pollen tube  
585 shank.

586 Our data suggests that NET2 proteins bind cortical F-  
587 actin at the membrane through association with PRK4  
588 and PRK5 at discrete foci, which we have termed 'actin-  
589 membrane contact sites (AMCSs)'. During this  
590 investigation, we determined that NET2s interact  
591 specifically with the PRK4/PRK5 subclade of PRKs (but  
592 not PRK1, PRK2, PRK3, or PRK6), in Y2H and FRET-  
593 FLIM assays. In growing pollen tubes PRK4 and PRK5  
594 localise to punctae in a similar distribution, specifically in  
595 the mature regions of the growing pollen tube, at which  
596 co-localisation with NET2A was observed. Therefore,  
597 NET2A interacts with PRK4 and PRK5 at the pollen tube  
598 plasma membrane at discrete foci.

599 In transient expression assays, it was noted that PRK4  
600 and PRK5 recruit NET2s to the plasma membrane: we  
601 therefore hypothesise that NET2s bind actin filaments at

602 the cell cortex through their associations with PRK4 and  
603 PRK5 at the pollen tube plasma membrane to form  
604 AMCSs. AMCSs appear to be persistent structures, and  
605 NET2A punctae were observed to localise permanently to  
606 the shank membrane, indicating their associations with  
607 PRKs to be highly stable. AMCSs formed by NET2-PRK  
608 interactions may therefore serve as stable membrane  
609 anchors for actin filaments, with roles in the organisation  
610 of cortical longitudinal actin cables in the pollen tube  
611 shank.

612 Through their associations with PRKs, the NET2  
613 subfamily may be implicated as having roles in  
614 extracellular signal transduction to the cytoskeleton  
615 during fertilisation. PRKs are believed to be important in  
616 fertilisation and transduce a number of extracellular  
617 signals to direct pollen tube growth to the female gamete.  
618 Notably, PRK4 and PRK5 recognise and transduce the  
619 extracellular signalling peptide, GRIM REAPER (GRI): an  
620 orthologue of *Lycopersicum esculentum* STIGMA-  
621 SPECIFIC 1 (LeSTIG1; Wrzaczek *et al.*, 2009), which  
622 promotes pollen tube growth downstream of binding  
623 tomato LePRK2 (Tang *et al.*, 2004; Huang *et al.*, 2014).  
624 During fertilisation, PRK4 and PRK5 may promote pollen  
625 tube growth in the stigma in response to binding  
626 members of the STIG1 family. Considering this, it is  
627 tempting to speculate that NET2A may regulate the actin  
628 cytoskeleton downstream of PRK4 and PRK5 to facilitate  
629 STIG1-stimulated pollen tube growth. Our data indicates  
630 that the kinase activity of PRK5 is important in promoting  
631 its interaction with NET2A. Consistent with this,  
632 phosphorylation of PiKIP1 by PiPRK1 has been shown to  
633 be important for interactions to occur between the two  
634 proteins (Skirpan *et al.*, 2001). It is therefore probable

635 that NET2A is phosphorylated by PRK5 and may serve  
636 as a downstream signalling effector. In Arabidopsis, other  
637 PRKs such as PRK2, PRK3 and PRK6 are believed to  
638 regulate cytoskeletal dynamics downstream of ligand  
639 binding to control pollen tube growth through the Rop  
640 signalling pathway, specifically at the pollen tube apex  
641 (Chang *et al.*, 2013; Zhao *et al.*, 2013; Takeuchi &  
642 Higashiyama, 2016). Importantly, here we have identified  
643 an additional mechanism by which unique PRKs may  
644 regulate the actin cytoskeleton through NET2A; distinct  
645 from apical Rop signalling and spatially localised to the  
646 shank region of the tube. We propose that PRK4 & PRK5  
647 may regulate the cortical longitudinal actin cables of the  
648 pollen tube shank in response to extracellular signals,  
649 during fertilisation.

650

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656

## 657 **Author Contributions**

658 PJH conceived the project, which was supervised by MJD  
659 and PJH. Most of the experiments were performed by  
660 PD, with exception of the cloning and expression of the  
661 NET2 NAB domains, generation of *pLAT52:FABD2-RFP*  
662 stable transgenic lines and rhodamine-phalloidin staining  
663 of *pNET2A:NET2A-GFP* pollen tubes (performed by  
664 MRD). Generation of *pNET2A:NET2A-GFP* stable  
665 transgenic lines was performed by MJD, and generation

666 of the PRK5<sup>K403R</sup> construct was performed by JK. PD  
667 prepared the figures and wrote the manuscript with MJD,  
668 TJH and PJH.

669

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847

## 848 **Figure Legends**

849 **Fig 1: NET2s belong to the NET superfamily of actin-**  
850 **binding proteins and localise to the actin**  
851 **cytoskeleton in *Nicotiana benthamiana* leaf epidermal**  
852 **cells through conserved N-terminal NET actin-**  
853 **binding (NAB) domains**

854 (a) multiple alignment of the NET superfamily NAB  
855 domains. (b) NET2A-NAB-GFP co-localises with actin  
856 filaments *in vivo*. (c) disruption of the actin cytoskeleton  
857 using 40  $\mu$ M Cytochalasin D results in breakdown of  
858 NET2A-NAB-GFP filament network. (d) GFP fusions of  
859 NET2B, NET2C and NET2D NAB domains also localise  
860 to actin filaments *in vivo*. (e) full-length NET2A-GFP co-  
861 localises with actin-filaments *in vivo*. Scale bar = 10  $\mu$ m.

862

863 **Fig 2: NET2A localises to punctae at the pollen tube**  
864 **plasma membrane that co-localise with actin**  
865 **filaments**

866 (a, b) subcellular localisation of natively expressed  
867 NET2A-GFP to the plasma membrane in growing

868 Arabidopsis pollen tubes (single z-plane images). (c) co-  
869 localisation of NET2A-GFP punctae with actin filaments in  
870 the Arabidopsis pollen tube shank, labelled with  
871 rhodamine-phalloidin. (d) co-localisation of NET2A-GFP  
872 punctae and the actin-marker, FABD2-RFP.  $80.2 \pm 6.1$  %  
873 of NET2A-GFP punctae were observed to co-localise with  
874 FABD2-RFP-labelled actin filaments. Scale bar = 10  $\mu$ m.

875

876 **Fig 3: NET2A interacts with Arabidopsis PRK4 and**  
877 **PRK5**

878 (a) NET2A interacts with PRK4 and PRK5 in yeast-2-  
879 hybrid assays. Yeast were grown on permissive media  
880 lacking Tryptophan and Leucine (-WL), or selective media  
881 lacking Tryptophan, Leucine and Histidine (-WLH). Yeast  
882 containing pGADT7-NET2A and pGBKT7-PRK4, or  
883 pGADT7-NET2A and pGBKT7-PRK5 were able to grow  
884 on selective media, indicating an interaction. Yeast  
885 containing pGADT7-NET2A and empty pGBKT7, empty  
886 pGADT7 and pGBKT7-PRK4, and empty pGADT7 and  
887 pGBKT7-PRK5 were used as negative controls and were  
888 unable to grow on selective media. (b) FRET-FLIM  
889 (Förster resonance energy transfer-fluorescence lifetime  
890 imaging microscopy) analysis of interactions between  
891 PRK4-GFP and NET2A-mCherry, and PRK5-GFP and  
892 NET2A-mCherry in *Nicotiana benthamiana* leaf epidermal  
893 cells. The average fluorescence lifetimes of the PRK4-  
894 GFP and PRK5-GFP donor constructs was reduced in  
895 the presence of the NET2A-mCherry acceptor construct,  
896 to comparable levels to the GFP-mCherry control. Images  
897 are pseudocoloured according to GFP fluorescence  
898 lifetime. Associated charts represent peak lifetime  
899 frequency of the acceptor construct in each image. A

900 leftward shift in peak lifetime frequency indicates a  
901 reduction in average GFP fluorescence lifetime. (c)  
902 diagrammatic representation of actin-membrane  
903 interactions mediated by NET2A and PRK4 & PRK5.  
904 Error bars on charts correspond to standard deviation.

905

906 **Fig 4: PRK4 and PRK5 interact with NET2A through**  
907 **their cytosolic kinase domains and recruit NET2A to**  
908 **the plasma membrane in *Nicotiana benthamiana* leaf**  
909 **epidermal cells.**

910 (a) co-expression of NET2A-GFP with PRK4-RFP or  
911 PRK5-RFP induces alterations in NET2A-GFP subcellular  
912 localisation in *N. benthamiana* transient assays. (b)  
913 NET2A-GFP co-localises with PRK5-RFP at the plasma  
914 membrane when both constructs are co-expressed  
915 together. (c) schematic diagrams of PRK4 $\Delta$ K (PRK4  
916 without the intracellular kinase domain) and PRK5 $\Delta$ K  
917 (PRK5 without the intracellular kinase domain) truncation  
918 mutants. (d) NET2A-GFP does not localise to the plasma  
919 membrane when co-expressed with PRK4 $\Delta$ K-RFP or  
920 PRK5 $\Delta$ K-RFP. (e) NET2A-GFP does not interact with  
921 PRK5 $\Delta$ K-RFP in FRET-FLIM (Förster resonance energy  
922 transfer-fluorescence lifetime imaging microscopy)  
923 interaction assays. (f) NET2A-GFP cannot be recruited to  
924 the membrane by PRK $\Delta$ K mutants. (g) FRET-FLIM  
925 indicates the interaction between NET2A-GFP and  
926 PRK5-RFP is weakened in the PRK5<sup>K403R</sup> mutant (PRK5  
927 with Lysine-403 replaced by Arginine). Scale bars: 10  $\mu$ m.  
928 Error bars on charts correspond to standard deviation.

929

930

931 **Fig 5: PRK4 and PRK5 localise to punctae at the**  
932 **plasma membrane of the pollen tube shank**

933 (a) PRK4-GFP in Arabidopsis pollen tubes. (i) max  
934 projection of whole pollen tube. (ii) magnified image of  
935 PRK4-GFP punctae at the pollen tube shank (cortical  
936 section). (iii) magnified image of PRK4-GFP punctae at  
937 the pollen tube shank (cross-section). (b) PRK5-GFP in  
938 Arabidopsis pollen tubes. (i) cross section of whole pollen  
939 tube. (ii) magnified image of PRK5-GFP punctae at the  
940 pollen tube shank (cortical section). (iii) magnified image  
941 of PRK5-GFP punctae at the pollen tube shank (cross-  
942 section). Scale bars: (i) = 10  $\mu\text{m}$ , (ii) and (iii) = 5  $\mu\text{m}$ .

943

944 **Figure 6: NET2A associates with PRKs at discreet**  
945 **foci at the shank plasma membrane of Arabidopsis**  
946 **pollen tubes**

947 (a) NET2A-GFP punctae co-localise with PRK4-RFP  
948 punctae in Arabidopsis pollen tubes. Scale bar = 10  $\mu\text{m}$ .  
949 (b) magnified image depicted by the inset in (a). Scale  
950 bar = 2  $\mu\text{m}$ .

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## 959    **Supporting Information Legends**

960

961    **Fig. S1: NET2B-GFP subcellular localisation in *N.***  
962    ***benthamiana* leaf epidermal cells.**

963    Full-length NET2B-GFP localises to actin filaments in a  
964    beads-on-a-string pattern characteristic of NET proteins  
965    when expressed in *N. benthamiana* leaf epidermal cells.  
966    Scale bar: 10  $\mu$ m.

967

968    **Fig. S2: Kymograph of video S1 showing co-**  
969    **ordinated linear movement of NET2A-GFP patches.**

970    The white line indicates the position of the kymograph  
971    which was taken over a width of 5 pixels.

972    (a) The kymograph shows movement initiating at  
973    approximately 270 s from time 0.

974    (b) (indicated by triangular arrowhead). Three  
975    neighbouring patches move in the retrograde direction. A  
976    3 pixel kymograph along the centre line of the pollen tube  
977    shows that the NET2A patch distribution persists over  
978    long time scales.

979    (c) Patches form in a zone behind the growing tip.  
980    Nascent patches initiating during the duration of the time  
981    series are located in a zone marked by an asterisk. The  
982    white-bordered scale bar is 20  $\mu$ m.

983

984

985

986 **Fig. S3: Interactions between NET2A and Arabidopsis**  
987 **PRKs are restricted to PRK4 and PRK5 in Y2H**  
988 **assays, and NET2A is unable to interact with PRK1,**  
989 **PRK2, PRK3 or PRK6.**

990 Yeast were grown on permissive (-WL) media, or  
991 selective (-WLH) media. Only yeast containing both  
992 pGADT7-NET2A and pGBKT7-PRK4 or pGBKT7-PRK5  
993 were able to grow on selective media, indicating an  
994 interaction. Yeast containing pGADT7-NET2A and  
995 pGBKT7-PRK1, pGBKT7-PRK2, pGBKT7-PRK3 or  
996 pGBKT7-PRK6 were unable to grow on selective media,  
997 indicating no interaction between these proteins.

998

999 **Fig. S4: NET2A-GFP is absent from transvacuolar**  
1000 **cytoplasmic strands when co-expressed with PRK5-**  
1001 **RFP in *N. benthamiana* leaf epidermal cells.**

1002 (a) cross-section of leaf epidermal cells expressing  
1003 NET2A-GFP alone. Arrows indicate NET2A-GFP  
1004 localising to transvacuolar cytoplasmic strands.

1005 (b) cross-section of leaf epidermal cells co-expressing  
1006 NET2A-GFP alongside PRK5-RFP. Scale bar: 10 µm.

1007

1008 **Fig. S5: NET2A-GFP localises to actin filaments when**  
1009 **co-expressed with PRK6-RFP in *N. benthamiana* leaf**  
1010 **epidermal cells.**

1011 NET2A-GFP is not recruited to the plasma membrane.  
1012 Scale bar: 10 µm

1013

1014

1015 **Fig. S6: NET2B-GFP is recruited to the plasma**  
1016 **membrane by PRK4-RFP and PRK5-RFP in *N.***  
1017 ***benthamiana* leaf epidermal cells, but not by PRK6-**  
1018 **RFP.**

1019 Scale bar: 10  $\mu$ m

1020

1021 **Table S1: Primers used in this study.**

1022

1023 **Table S2: NET2A does not interact with PRK1, PRK2,**  
1024 **PRK3 or PRK6 in FRET-FLIM assays.**

1025 Each PRK was expressed as a GFP fusion in *N.*  
1026 *benthamiana* either alone, or with NET2A-mCherry. The  
1027 average fluorescence lifetimes of PRK1-GFP, PRK2-  
1028 GFP, PRK3-GFP and PRK6-GFP were not significantly  
1029 reduced by NET2A-mCherry, indicating no interaction  
1030 between NET2A and each PRK.  $\pm$  = standard deviation  
1031 of mean values. ns = nanoseconds.

1032

1033 **Table S3: NET2B interacts specifically with PRK4 and**  
1034 **PRK5 in FRET-FLIM assays but not with PRK1, PRK2,**  
1035 **PRK3 or PRK6.**

1036 NET2B was expressed alone, or with RFP-fluorophore  
1037 fusions of each PRK. PRK4-RFP and PRK5-RFP were  
1038 observed to reduce the fluorescence lifetime of NET2B-  
1039 GFP, indicative of an interaction. The average  
1040 fluorescence lifetime of NET2B-GFP was not decreased  
1041 by PRK1-RFP, PRK2-mCherry, PRK3-RFP and PRK6-  
1042 RFP indicating no interaction occurred.  $\pm$  = standard  
1043 deviation of mean values. ns = nanoseconds.

1044

1045

1046 **Video S1: NET2A-GFP Punctae Dynamics in Growing**  
1047 **Pollen Tubes.**

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1050

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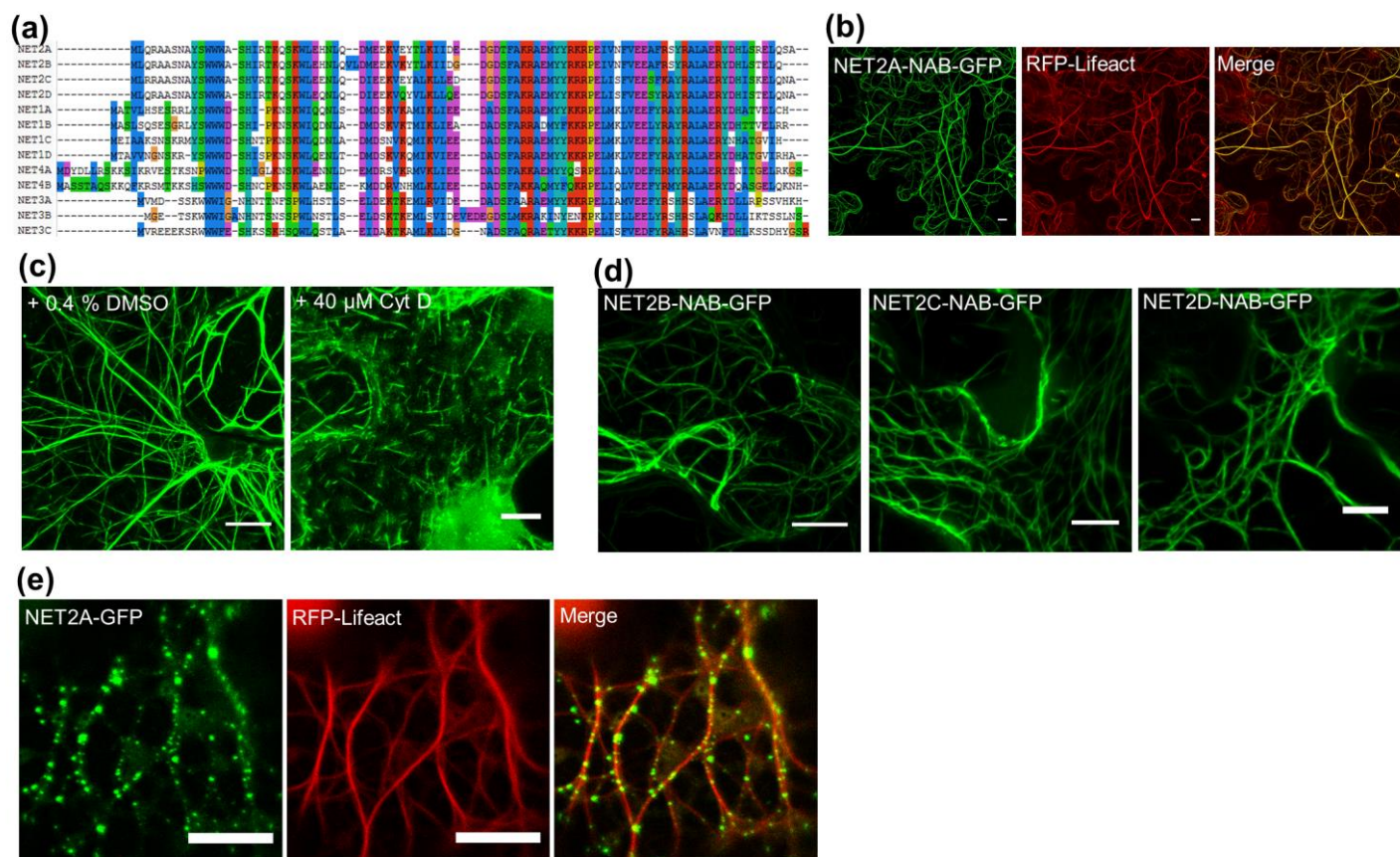
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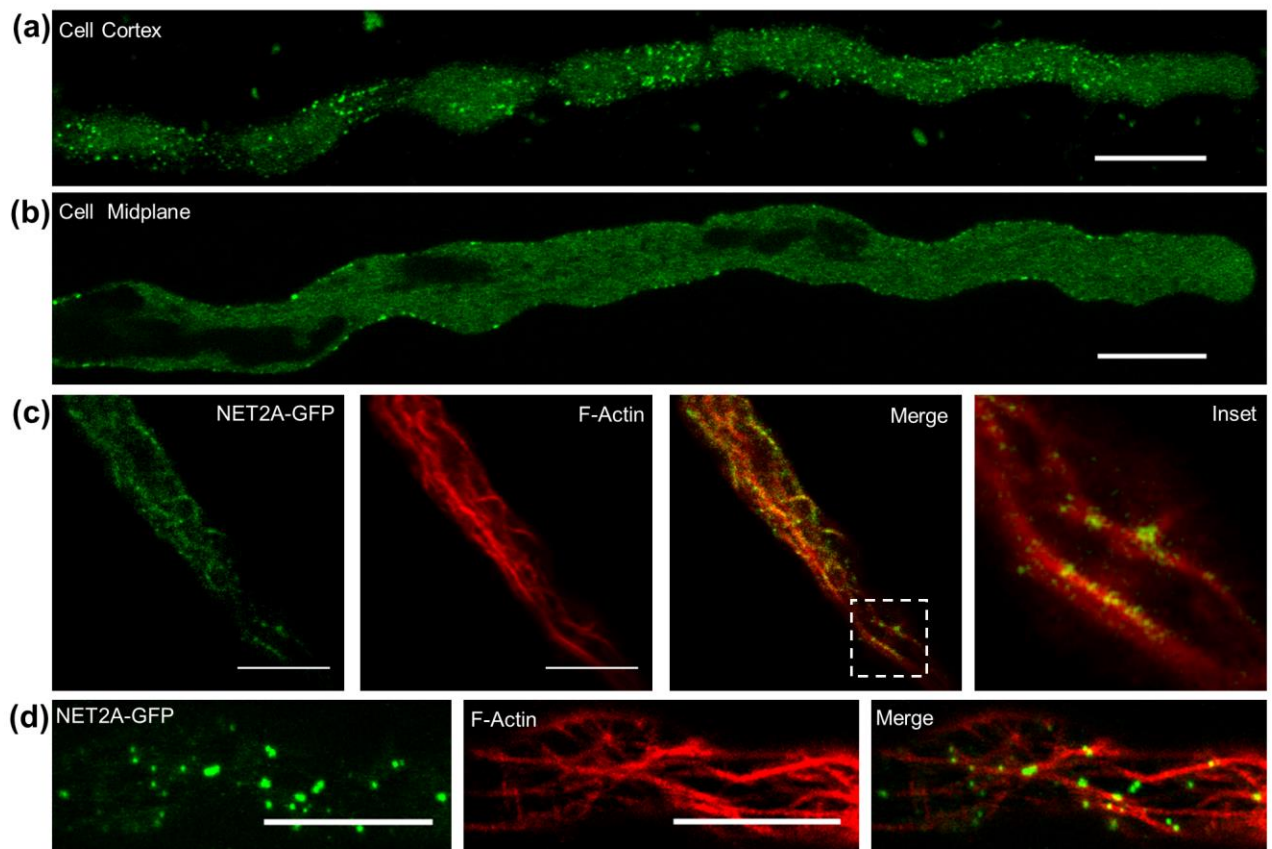
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# Figures

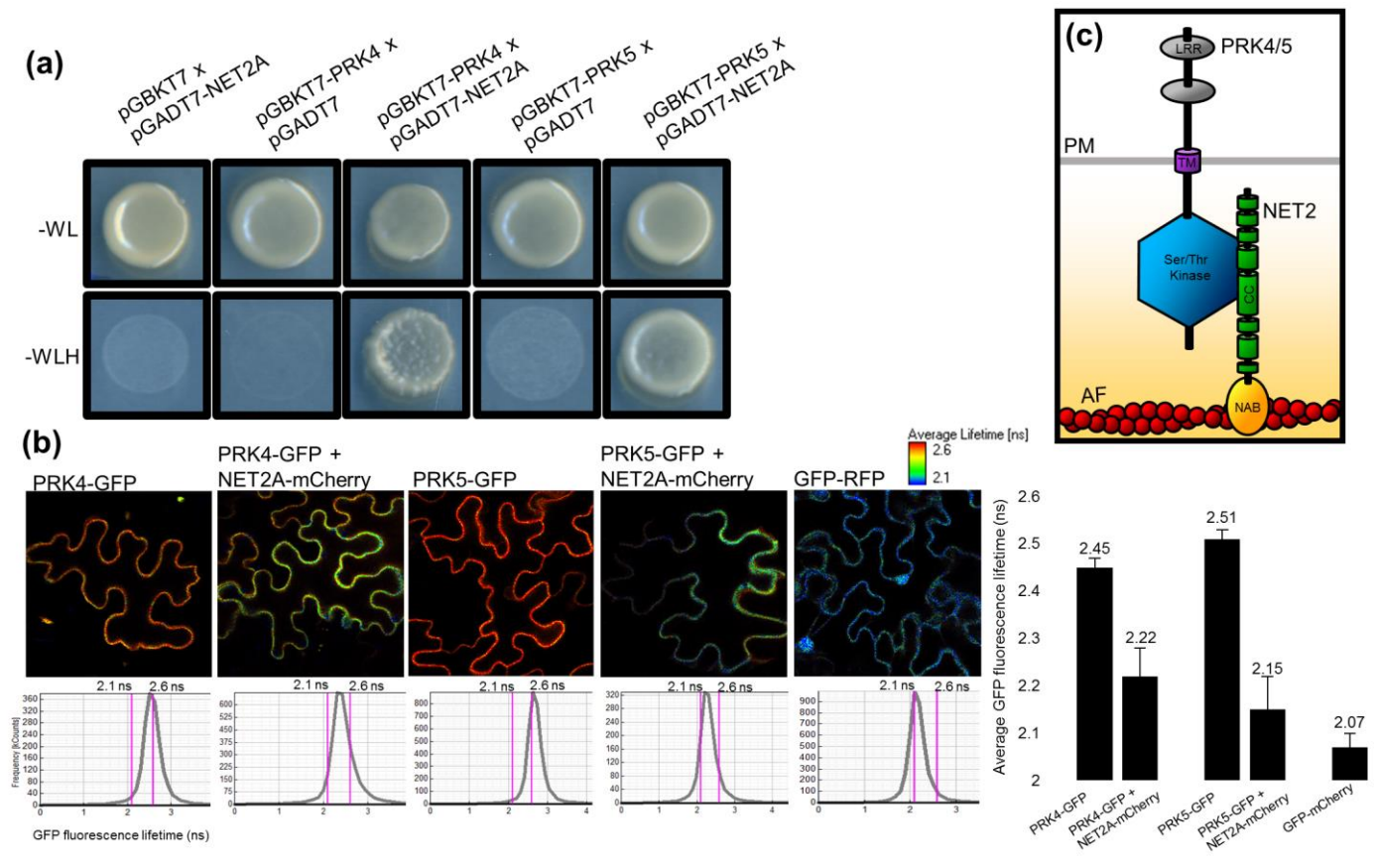
Fig. 1



**Fig. 2**

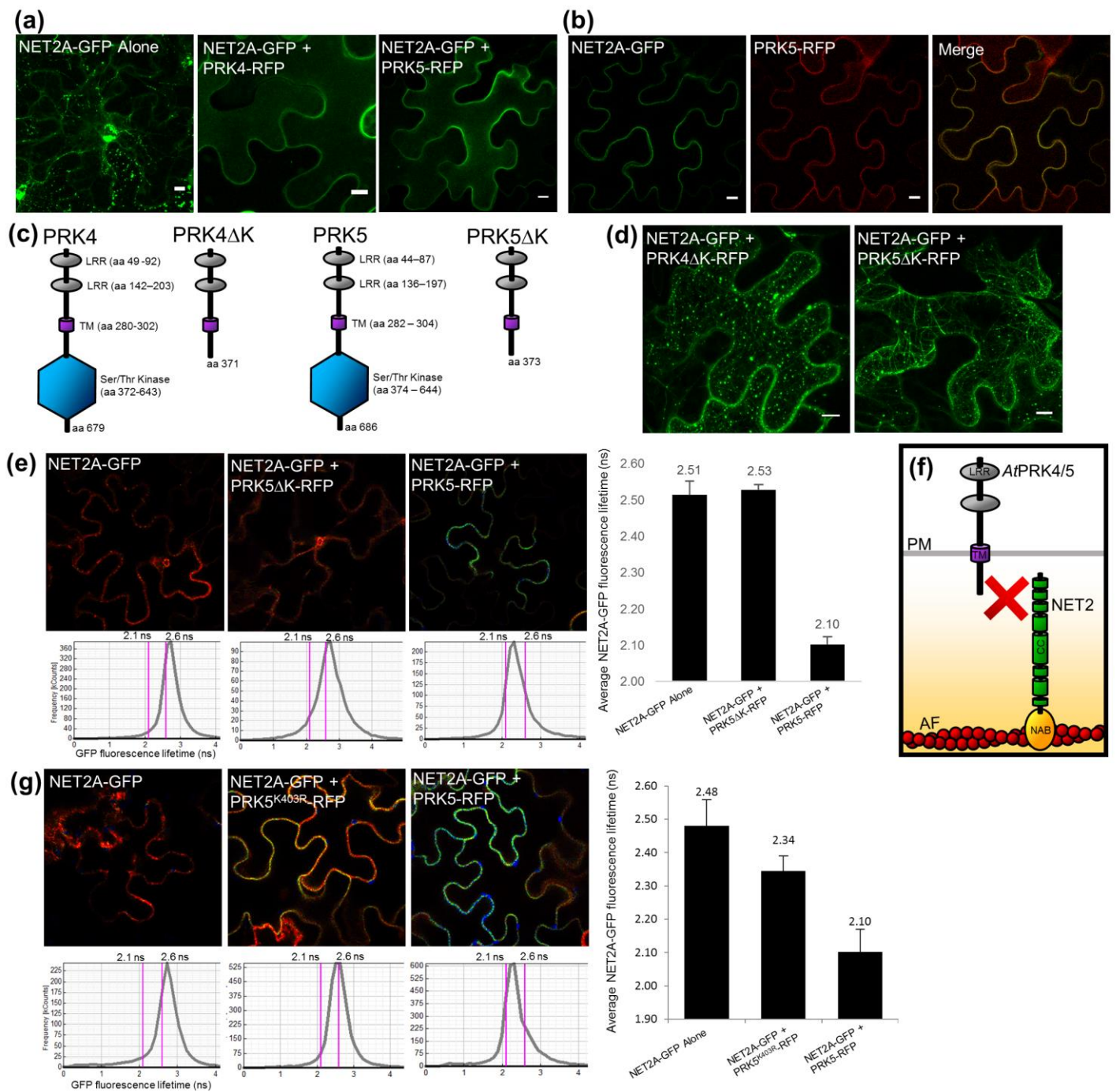


**Fig. 3**





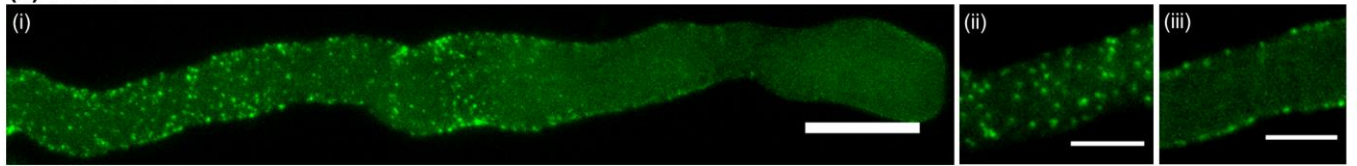
**Fig. 4**



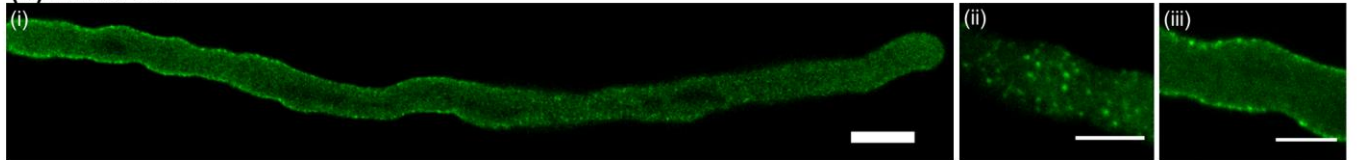


**Fig. 5**

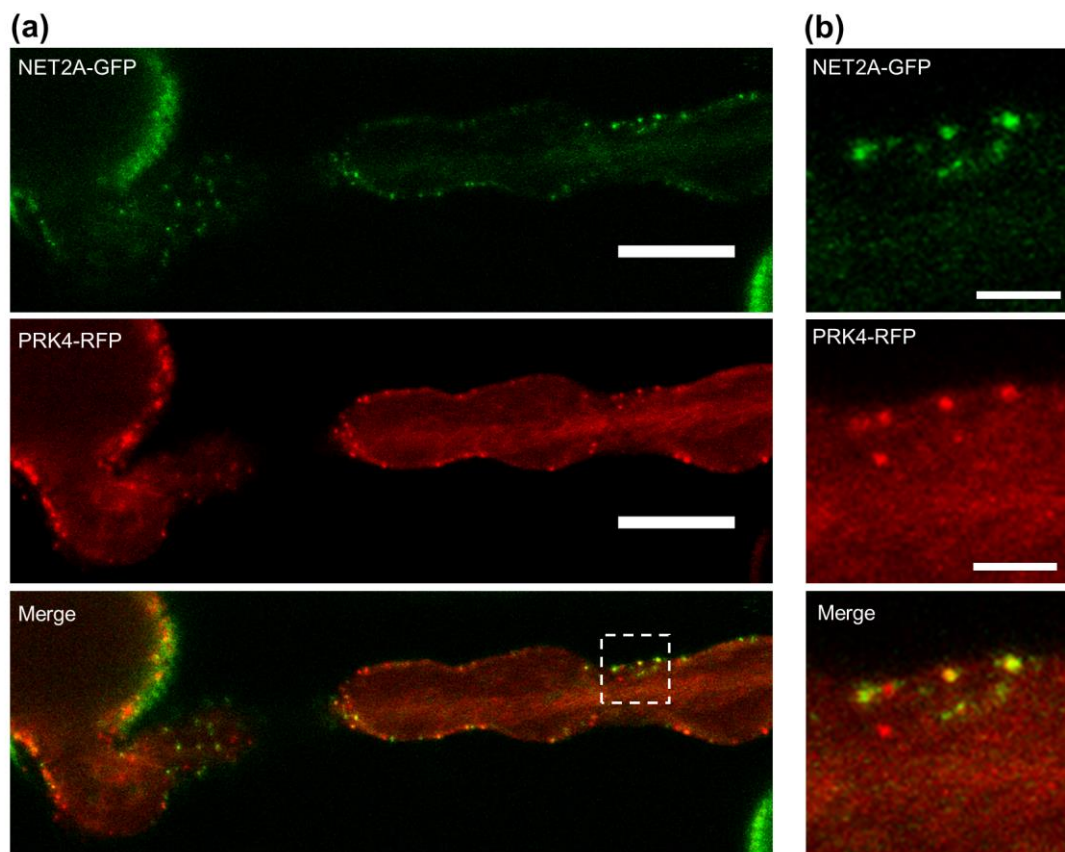
**(a) PRK4-GFP**



**(b) PRK5-GFP**



**Fig. 6**



## Supplemental Information

Fig. S1

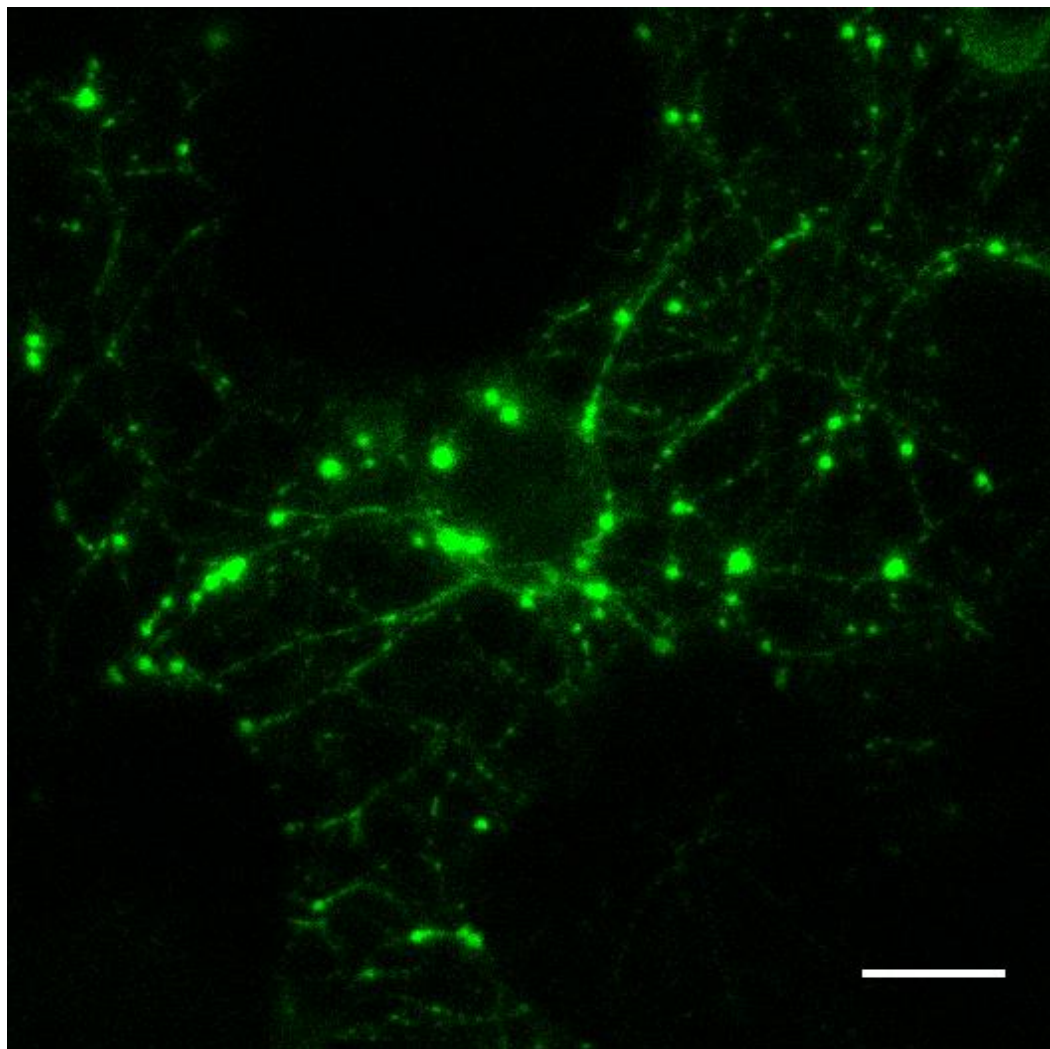
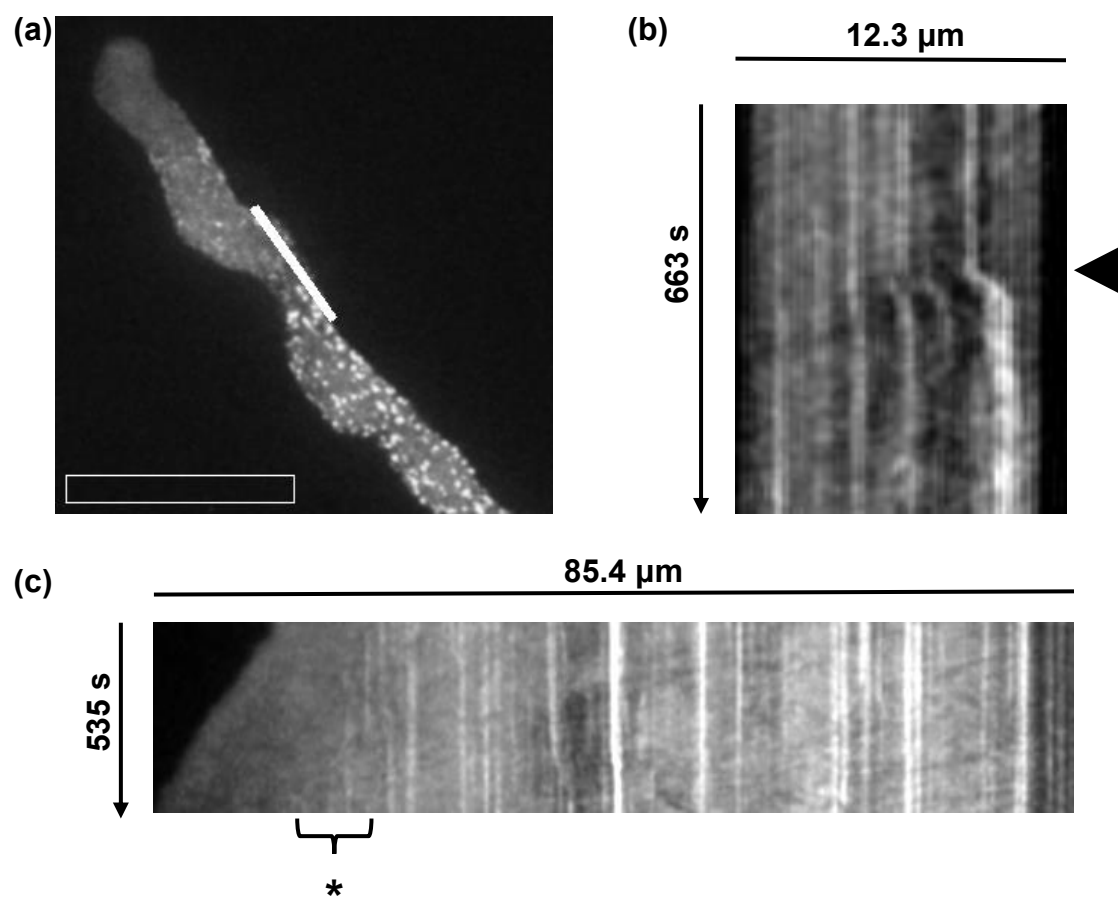
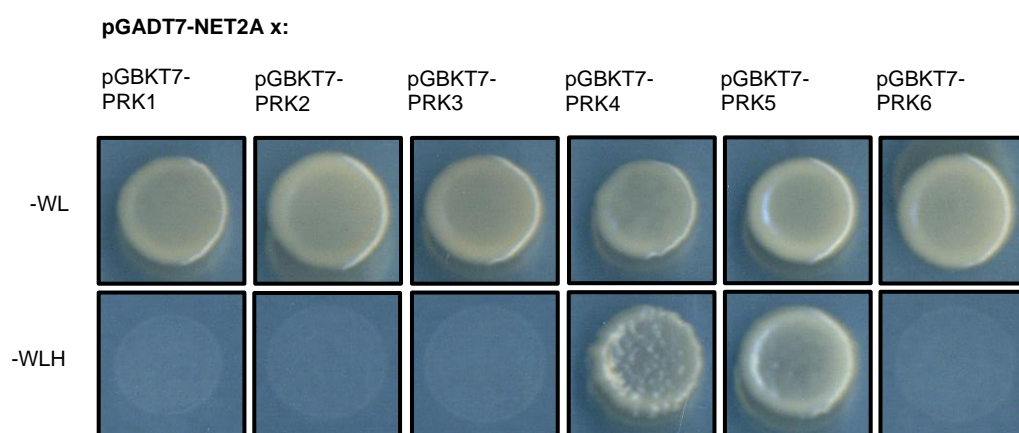


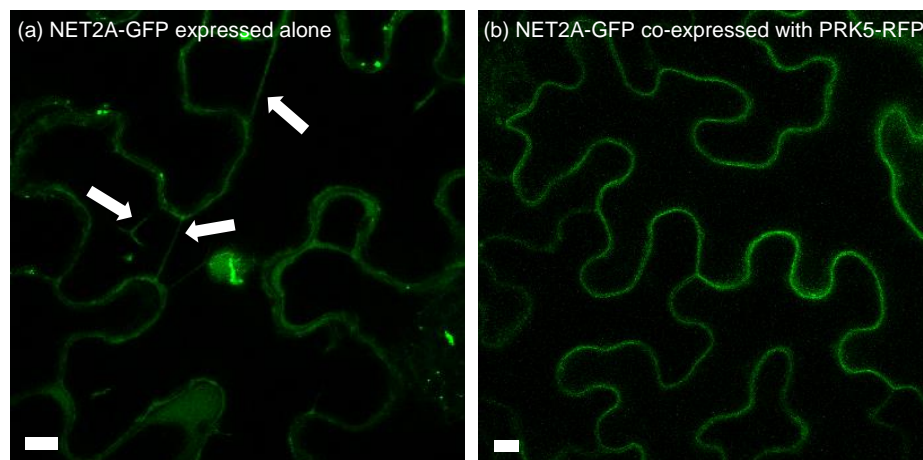
Fig. S2



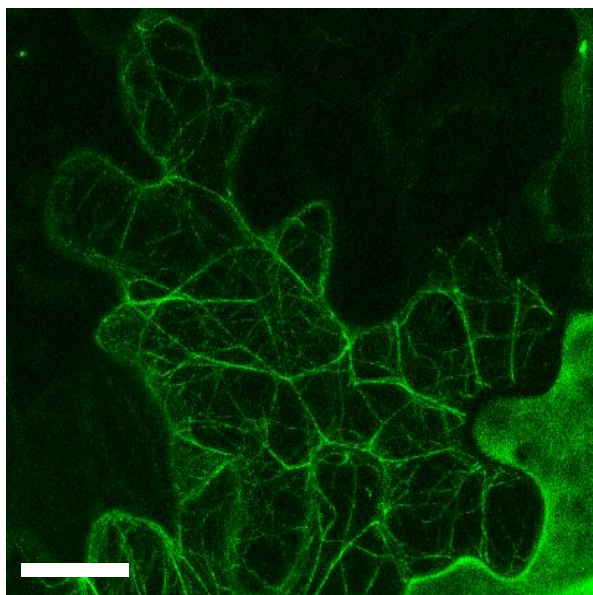
**Fig. S3**



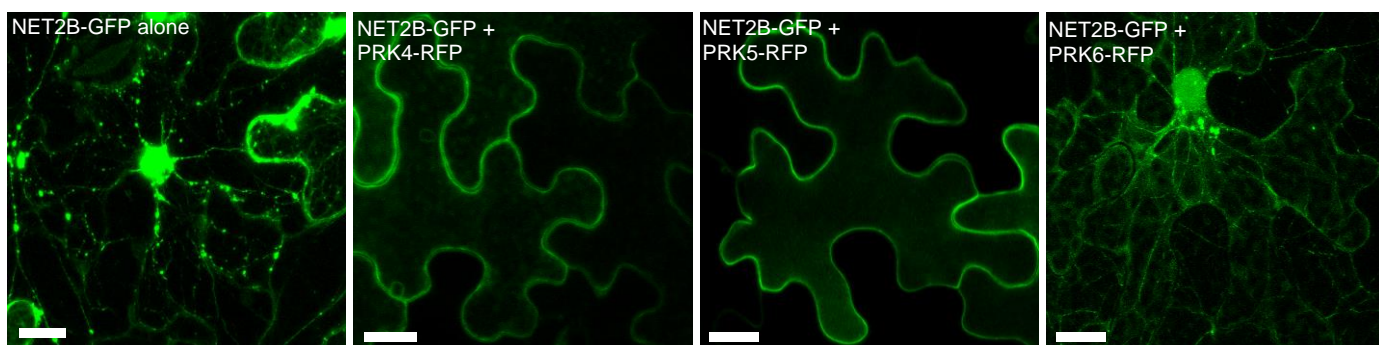
**Fig. S4**



**Fig. S5**



**Fig. S6**





### Table S1

Construct	Forward/Reverse	Primer Sequence 5'→3'
Full-length NET2A	F	GGGGACAAGTTTGTACAAAAAAGCAGGCTTCACCATGTTGCAGAGAGCAGCGAGC
	R	GGGGACCACTTTGTACAAGAAAGCTGGGTCTTCAGGGAGCTTCCCAGGTG
Full-length NET2B	F	GGGGACAAGTTTGTACAAAAAAGCAGGCTTCACCATGTTGCAGAGAGCAGCGAGC
	R	GGGGACCACTTTGTACAAGAAAGCTGGGTCTGCTTTTGCATATTCAGG
Full-length PRK1	F	GGGGACAAGTTTGTACAAAAAAGCAGGCTTCACCATGCCTCCATGCAGGCG
	R	GGGGACCACTTTGTACAAGAAAGCTGGGTCTGCAAAGCTGATACTCTC
Full-length PRK2	F	GGGGACAAGTTTGTACAAAAAAGCAGGCTTCACCATGGAATCCAAATGTCATGTTGG
	R	GGGGACCACTTTGTACAAGAAAGCTGGGTCTGACAAGTTAATTCCTCAC
Full-length PRK3	F	GGGGACAAGTTTGTACAAAAAAGCAGGCTTCACCATGACTGCTGTCTATTT
	R	GGGGACCACTTTGTACAAGAAAGCTGGGTCAAGTGTACTCGTTCAT
Full-length PRK4	F	GGGGACAAGTTTGTACAAAAAAGCAGGCTTCACCATGCTAACTGGGAGACC
	R	GGGGACCACTTTGTACAAGAAAGCTGGGTCTCGATTTCATGGCGAAACC
Full-length PRK5	F	GGGGACAAGTTTGTACAAAAAAGCAGGCTTCACCATGCGCAATTGGGAGGAC
	R	GGGGACCACTTTGTACAAGAAAGCTGGGTCTCGATTTCATCGAGAAACCA
Full-length PRK6	F	GGGGACAAGTTTGTACAAAAAAGCAGGCTTCACCATGGCTGCTGCTGTTCTG
	R	GGGGACCACTTTGTACAAGAAAGCTGGGTCTGAGTTTCTGTTCTATCCTTCTA
NET2B-NAB (NET2B <sup>1-93</sup> )	F	GGGGACAAGTTTGTACAAAAAAGCAGGCTTCACCATGTTGCAGAGAGCAGCGAGC
	R	GGGGACCACTTTGTACAAGAAAGCTGGGTCTTGAAGTTCTGTGGACAAATGATC
NET2C-NAB (NET2C <sup>1-93</sup> )	F	GGGGACAAGTTTGTACAAAAAAGCAGGCTTCACCATGCTACGAAGAGCTGCGAGC
	R	GGGGACCACTTTGTACAAGAAAGCTGGGTCTGAGTTTGAAGCTCTTTAGAG
NET2D-NAB (NET2D <sup>1-93</sup> )	F	GGGGACAAGTTTGTACAAAAAAGCAGGCTTCACCATGCTGCAACGAGCTGCGAGTAATG
	R	GGGGACCACTTTGTACAAGAAAGCTGGGTCTGAGTTTGAAGCTCTGTAG
PRK4ΔK (PRK4 <sup>1-374</sup> )	F	GGGGACAAGTTTGTACAAAAAAGCAGGCTTCACCATGCTAACTGGGAGACC
	R	GGGGACCACTTTGTACAAGAAAGCTGGGTCTGAGGCTCTAAGAAGATCTTGAAGG
PRK5ΔK (PRK5 <sup>1-376</sup> )	F	GGGGACAAGTTTGTACAAAAAAGCAGGCTTCACCATGCGCAATTGGGAGGAC
	R	GGGGACCACTTTGTACAAGAAAGCTGGGTCTGAGTCTCTCAAAGATCTTGAAGATCG
5'AscI-mCherry-3'BstBI	F	CTTGGCGCGCCATGGTGAGCAAGGGCGAGGAGGATAA
	R	TCTTTCGAATTACTTGTACAGCTCGTCCATGCC
5'SacI-pLAT52-3'SpeI	F	TCAGGAGCTCTTGAGGAATGATCGATTCTGG
	R	CCATACTAGTGAATTTTTTTTTTGGTGTGTG
PRK5 <sup>K403R</sup>	F	GTGGACAAACATTGGTTGTGAGGAGGTATAACATATGAACAATG
	R	CATTGTTTCATATGTTTATACCTCCTCACACCAATGTTTGTCCAC
NET2A Y2H	F	GGGGACAAGTTTGTACAAAAAAGCAGGCTTCACCATGTTGCAGAGAGCAGCGAGC
	R	GGGGACCACTTTGTACAAGAAAGCTGGGTCTTATTCAGGGAGCTTCCCAGGTG
PRK1 Y2H	F	GGGGACAAGTTTGTACAAAAAAGCAGGCTTCACCATGCCTCCATGCAGGCG
	R	GGGGACCACTTTGTACAAGAAAGCTGGGTCTGCAAAGCTGATACTCTC

PRK2 Y2H	F	GGGGACAAGTTTGTACAAAAAAGCAGGCTTCACCATGGAATCCAAATGTCTCATGTTTCG
	R	GGGGACCACTTTGTACAAGAAAGCTGGGTCTGACAAGTTAATCCCTCAC
PRK3 Y2H	F	GGGGACAAGTTTGTACAAAAAAGCAGGCTTCACCATGACTGCTGTTCTATTT
	R	GGGGACCACTTTGTACAAGAAAGCTGGGTCAAGTGTTACTCGTTCTAT
PRK4 Y2H	F	GGGGACAAGTTTGTACAAAAAAGCAGGCTTCACCATGCTAACTGGGAGACC
	R	GGGGACCACTTTGTACAAGAAAGCTGGGTCTCGATTCATGGCGAAACC
PRK5 Y2H	F	GGGGACAAGTTTGTACAAAAAAGCAGGCTTCACCATGCGCAATTGGGAGGAC
	R	GGGGACCACTTTGTACAAGAAAGCTGGGTCTCGATTCATCGAGAAACCA
PRK6 Y2H	F	GGGGACAAGTTTGTACAAAAAAGCAGGCTTCACCATGGCTGCTGCTGTTCTG
	R	GGGGACCACTTTGTACAAGAAAGCTGGGTCAAGTTTTACTTGTTCTATCCTTCTA

**Table S2**

Donor Construct	Mean Donor GFP Fluorescence Lifetime			
	Expressed without NET2A-mCherry	n	Co-expressed with NET2A-mCherry	n
PRK1-GFP	2.50 ± 0.03 ns	6	2.49 ± 0.02 ns	6
PRK2-GFP	2.57 ± 0.02 ns	10	2.54 ± 0.03 ns	10
PRK3-GFP	2.47 ± 0.02 ns	10	2.52 ± 0.02 ns	10
PRK6-GFP	2.43 ± 0.04 ns	10	2.43 ± 0.07 ns	10
GFP-mCherry	2.07 ± 0.03 ns			12

**Table S3**

Constructs Expressed	Mean GFP Fluorescence Lifetime (ns)	n
NET2B-GFP	2.50 ± 0.04	10
NET2B-GFP + PRK1-RFP	2.46 ± 0.03	10
NET2B-GFP + PRK2-mCherry	2.50 ± 0.04	10
NET2B-GFP + PRK3-RFP	2.48 ± 0.02	10
NET2B-GFP + PRK4-RFP	2.22 ± 0.14	10
NET2B-GFP + PRK5-RFP	2.14 ± 0.09	10
NET2B-GFP + PRK6-RFP	2.47 ± 0.06	10
GFP-RFP	1.99 ± 0.04	5
GFP-mCherry	2.03 ± 0.05	5